An Antifeedant for Fall Armyworm Larvae From Neem Seeds

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AN ANTIFEEDANT FOR FALL ARMYWORM LARVAE FROM NEEM SEEDS

By J. D. Warthen, Jr., R. E. Redfern, E. C. Uebel, and G. D. Mills, Jr.[1/]

ABSTRACT

A compound from an ethanolic extract of neem seeds, Azadirachta indica A. Juss., possessed significant antifeedant activity for fall armyworm first-instar larvae at 0.35 parts per million in an artificial diet. The compound deterred feeding and therefore caused an 88 percent reduction in weight of the larvae without mortality. The isolated compound was identified as azadirachtin, a triterpenoid that had been shown to have antifeedant activity against several other insects. Techniques of open-column reversed-phase chromatography and high-performance liquid chromatography (with recycle), not reported before, were used for isolation.

KEYWORDS: Azadirachta indica, neem, azadirachtin, antifeedant, Spodoptera frugiperda, fall armyworm.

INTRODUCTION

The neem tree, Azadirachta indica A. Juss.,[2/] has been studied extensively because it has long been used in India and Africa for its insect repellent and medicinal properties (9, 10, 11). The triterpenoids meliantriol (6) and azadirachtin (2, 3, 4, 14) have been isolated from neem. Meliantriol, obtained from the seed oil of the fruit, deterred feeding by the desert locust, Schistocerca gregaria (Forskal) (6). Azadirachtin, obtained from the leaves and seeds, deterred feeding by this insect and by larvae of the diamondback moth, Plutella xylostella (L.); cabbage white, Pieris brassicae (L.); tobacco budworm, Heliothis virescens (F.); cotton stainer, Dysdercus suturellus (Herrich-Schäffer); greater wax moth, Galleria mellonella (L.); and termite, Reticulitermes sp. (9, 13, 14).

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2/ Also referred to as Melia azadirachta L., M. indica Brandis, Margosa tree, or Indian lilac.

A systemic effect for azadirachtin was demonstrated by Gill and Lewis (5). Young bean plants grown in soil that was treated with azadirachtin showed little damage by S. gregaria. Bean seedlings grown from seeds soaked in azadirachtin solutions were protected against damage by S. gregaria adults for 1 week after germination in cage feeding tests. Systemic uptake without phytotoxicity has also been demonstrated in wheat, barley, rice, sugarcane, grass, tomato, cotton, chrysanthemum, and the small spindle tree (11). Thus, neem is of considerable interest as a source of antifeedants that can be used as an alternative means of pest control and in integrated pest management programs.

Leaf extracts of the closely related M. azedarach L. (chinaberry tree, Persian lilac), when incorporated into an artificial diet, deterred feeding in fall armyworm larvae, but the active component was never determined (7). Morgan and Thornton (8) suggested that it might be azadirachtin, but this is contrary to the hypothesis of Ruscoe (13). Bioassays with neem seed extracts in an artificial diet (12) clearly demonstrated significant antifeedant activity for first-instar larvae of the fall armyworm, Spodoptera frugiperda (J. E. Smith). We therefore isolated and identified the compound responsible for this activity.

MATERIALS AND METHODS

Plant Material

Fresh neem seeds (5.76 kg) from India were placed in a large Waring Blendor, covered with 95 percent ethanol, and ground for 5 minutes. Grinding was then repeated with a fresh batch of 95 percent ethanol. When the ethanolic extracts were combined and filtered through a Büchner funnel, a yellow fatty layer separated out of the filtrate. The ethanolic extract was removed from the fat layer and evaporated to dryness in vacuo to give 375.9 g of material.

Solvents

All solvents used for extraction and open-column fractionation were reagent-grade. Methanol for high-performance liquid chromatography (hplc) was hplc-grade.

Partitioning

The solvent-free extract (375.9 g) was partitioned between 3800 ml of 95 percent methanol and 2500 ml of hexane. Each separated layer was then extracted with 600 ml of fresh opposing solvent, and the combined extracts were evaporated in vacuo to give 250 g of methanol-soluble and 168.1 g of hexane-soluble fractions. Since antifeedant activity for fall armyworm larvae was found only in the methanolic-soluble portion, it was chromatographed on a 98- by 8-cm o.d. column of 3 kg Florex RVM (Floridin, 60-100 mesh) slurry-packed in toluene. The fraction (250 g) was placed on the column in 1000 ml of toluene, and thirty 600-ml fractions were collected from the column via elution with 5 percent acetone-ether (3).

Bioassay

Fall armyworm larvae from our stock culture were laboratory-reared on an artificial diet (12). The test fractions for antifeedant activity were incorporated into 100 g of modified Burger (1) diet at the appropriate concentrations (table 1). Treated diets were poured into 1-oz jelly cups at the rate of 8 g/cup and allowed to cool to room temperature. Check diet cups were

Table 1.—Modified Burger (1) diet used to rear the fall armyworm larvae

Ingredient[1/]	Concentration
Water	800 ml
Potassium hydroxide (4M)	15 ml
Vitamins[2/]	12 ml
Choline chloride (15 percent)	25 ml
Acetic acid (25 percent)	40 ml
Formaldehyde (10 percent)	15 ml
Raw linseed oil	8.5 ml
Casein (vitamin free)	126 g
Sucrose	126 g
Wheat germ	108 g
Wesson salt "W"	36 g
Alphacel	18 g
Methyl 4-hydroxybenzoate	11 g
L-Ascorbic acid	15 g
Aureomycin	500 mg
Agar (60 g) in 2300 ml water[3/]	—

1/ The ingredients were added to a 1-gal Waring Blendor in the order shown.

2/ Contains the following vitamins/liter in water: calcium pantothenate, 12.0 g; niacin, 6.0 g; riboflavin, 3.0 g; folic acid, 3.0 g; thiamine HCL, 1.5 g; pyridoxine HCL, 1.5 g; biotin, 0.12 g; B_{12}, 0.006 g.

3/ Agar is added to cold water and brought to a rolling boil before being placed in the blender

untreated. One first-instar larva was then placed in each cup (10 cups were set up for each concentration). The cups were then capped with prepunched lids and placed in a holding room at 27° ± 1° with a 50 ± 5 percent relative humidity. The larvae were observed for mortality and were weighed.

Chromatography and Isolation Procedure

A sample (1.2 g) of the most active fractions (11-30, 7.6 g) from the Florex column was placed on a 100-g, 67.5- by 2.4-cm i.d., C-18 Phase-Bonded Hi-Flosil column (Applied Science Laboratories, Inc., 80-100 mesh). Eight 200-ml fractions were eluted successively with 40, 45, 50, 55, 60, 70, and 80 percent methanol-water and then with methanol. The third (73.3 mg) and fourth (116.3 mg) fractions were the most active. These fractions were placed again on the Hi-Flosil column and eluted successively to give three fractions (200 ml

each) with 50 percent, two fractions (200 ml each) with 55 percent, and one fraction (200 ml) with 60 percent methanol-water. The third (89.6 mg) and fourth (65.6 mg) fractions were the most active. These fractions were again placed on the Hi-Flosil column and eluted successively to give one 400-ml and eight 200-ml fractions with 50 percent methanol-water and one fraction (280 ml) from linear programing to straight methanol over 40 min. The third (18.0 mg), fourth (21.2 mg), and fifth (17.2 mg) fractions, designated A, B, and C, respectively, were the most active.

Each of these three fractions was subjected to hplc on a reversed-phase 30- by 0.78-cm i.d. μ Bondapak C-18 column with 50 percent methanol-water at 4 ml/min. Twenty-six 4-ml fractions were collected from each sample. A Waters Associates ALC-100 liquid chromatograph equipped with an M-6000 pump, a U6K injector, an M-660 solvent programer, a Schoeffel Instrument Corp. SF 770 multi-wavelength UV detector, and a Buchler Fractomette 200 fraction collector was used for all hplc. Monitoring at 217 nm was done for all hplc runs. Fractions A-16 and A-17, B-16 and B-17, and C-16 resulting from A, B, and C, respectively, were active.

An aliquot (5.55 percent) of A-16 was injected onto a 30- by 0.39-cm i.d. μ Bondapak C-18 column with 50 percent methanol-water at 1 ml/min as the eluent. The unknown peak was recycled twice and then collected on the third pass through the column. An authentic sample of azadirachtin (35.5 μg) was injected under the same conditions, also recycled twice, and collected on the third pass through the column.

Mass Spectral Analyses

This unknown from neem and the authentic azadirachtin, both purified by hplc recycle, were subjected to direct probe (180°) mass spectral analyses on an LKB 2091 at 70 eV and 50 μA ionizing current. The mass spectrum of the unknown was examined, and the intensities from mass to charge (m/e) 459-702 were manually measured. Peak m/e 559 was adjusted to 100 percent intensity, and all other intensities were adjusted accordingly. A spectrum was synthesized from these data via the Hewlett-Packard 5992A GC/MS Spectral Manipulation Program (cartridge 05992-10001) on their 9825A calculator.

RESULTS AND DISCUSSION

The fractions obtained from the Florex column and authentic azadirachtin were examined by thin-layer chromatography (tlc) on silica gel (Eastman Chromagram sheets, 100-μm thickness, acetone as developer). From the very beginning of our fractionation scheme, the greatest antifeedant activity was in fractions that contained a spot with an R_f identical to that of authentic azadirachtin. Hplc on the 0.39-cm i.d. μ Bondapak C-18 column was also used to compare fractions from the Hi-Flosil and the 0.78-cm i.d. μ Bondapak C-18 columns with the authentic azadirachtin. Again the greatest antifeedant activity always was in fractions containing a major peak at the R_V of authentic azadirachtin.

It appeared that the antifeedant activity was actually caused by azadirachtin. The unknown peak, which was collected via recycle after three

passes through the 0.39-cm i.d. μ Bondapak C-18 column, and the authentic azadirachtin, which was collected by the same procedure, had identical retention volumes. The ultraviolet absorption of both was always at a maximum of 217 nm. The techniques of open-column reversed-phase chromatography and hplc (with recycle) were used in place of extensive preparative tlc (2, 14) for the isolation of this antifeedant.

Mass spectral analyses of the unknown and authentic azadirachtin showed identical spectra. The mass spectral fragments reported by Butterworth et al. (4), which included the m/e 702 (M-18) peak, were apparent in our spectra. The synthesized spectrum (fig. 1) of the unknown shows the 200 highest mass units, which are the most important. The only other peaks mentioned by Butterworth et al. (4) that are not shown in figure 1 are those at m/e 100, 83, 55, and a metastable at 36.5. Our spectra also contained these ions with the metastable at 36.6.

Therefore, we conclude that our unknown is azadirachtin (fig. 2). No attempts were made to estimate the amount of azadirachtin present in our samples of neem seeds since this has been explored (3, 14).

Since all of the impure fractions in table 2 contained roughly the same amount of azadirachtin, by peak height measurement at 217-nm absorption, we carried out a dilution study to determine the concentration at which maximum antifeedant activity occurred without mortality. We found that 5 p/m in the larval diet, based upon milligram equivalents (in reference to milligrams of A, B, and C), produced the greatest decrease in feeding without mortality.

Table 2.—Bioassay against first-instar fall armyworm larvae of antifeedant fractions A-16, A-17, B-16, B-17, and C-16 over a range of concentrations

Fraction[1/]	Aliquot in μl	Mg equivalents in diet[2/]	P/M based on mg equivalents	Average weight in mg after 9 days[3/]
B-16[4/]	9.4	0.05	0.5	103
B-17[4/]	18.8	0.1	1	80
A-16[5/]	22.2	0.2	2	79
A-17[5/]	55.5	0.5	5	28
C-16[6/]	232.4	1.0	10	1.67[7/]
Check	—	—	—	269

1/ 4-ml volume.
2/ Per 100 g larval diet.
3/ From date put on media; 0 percent mortality; 10 replicates of each bioassay.
4/ From 21.2 mg of B.
5/ From 18.0 mg of A.
6/ From 17.2 mg of C.
7/ 100 percent mortality by the 13th day.

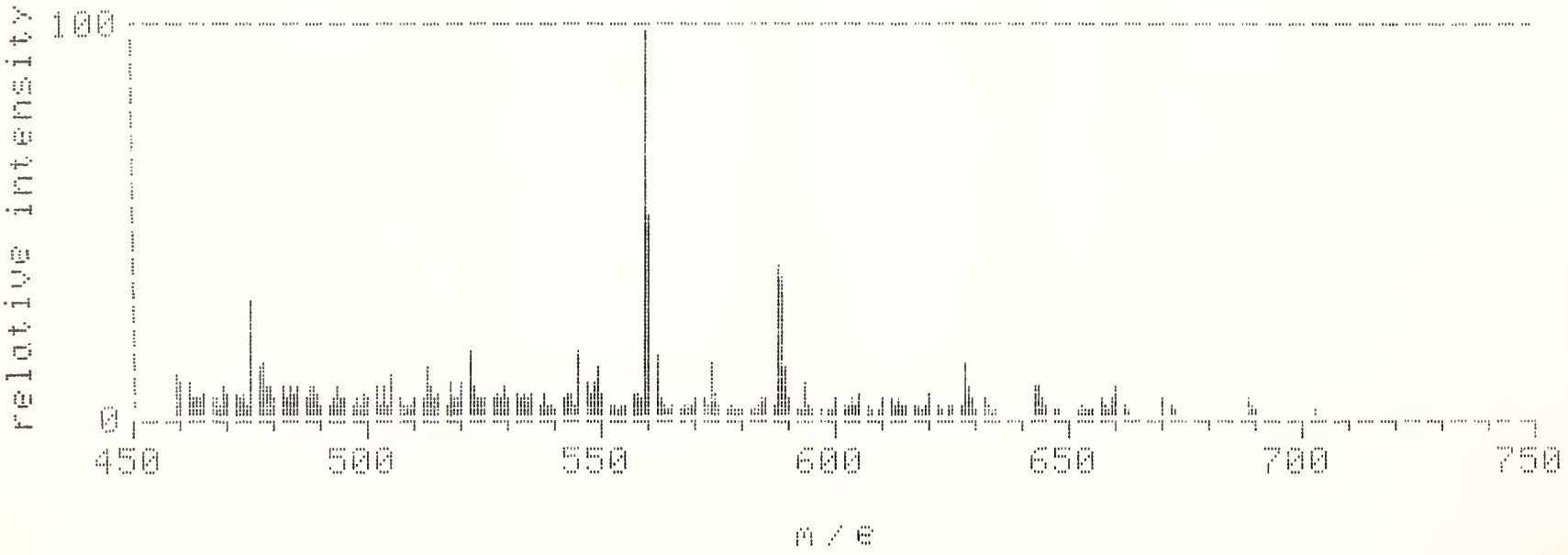

Figure 1.—Computer-generated electron impact mass spectrum of the 200 highest mass units of antifeedant from neem.

Table 3 shows the results of testing fractions from recycled A-16 at this 5-p/m concentration (0.5 mg equivalent/100 g diet in reference to milligrams

Table 3.—Bioassay for antifeedant activity against first-instar fall armyworm larvae of fractions from hplc recycled A-16 and authentic azadirachtin

Fraction[1/]	Aliquot in ml[2/]	Average weight in mg after 8 days[3/]
1-14	7.0	138
15	0.5	165
18	0.5	170
19-31	6.5	127
32-34	1.5	113
51-57	3.5 (actual p/m = 0.35)[4/]	26
Authentic azadirachtin[5/]	— (0.35 p/m)	29
Check	—	220

1/ Each numbered fraction contains 1-ml eluate from a 222-µl injection of A-16 (4-ml fraction).

2/ 0.5 mg equivalents/100 g larval diet $\cong$ 5 p/m.

3/ From date put on media; 0 percent mortality; 10 replicates of each bioassay.

4/ Based upon 217-nm absorption peak height in hplc runs vs. same of 35.5 µg authentic azadirachtin.

5/ 35.5 µg/100 g larval diet = 0.35 p/m.

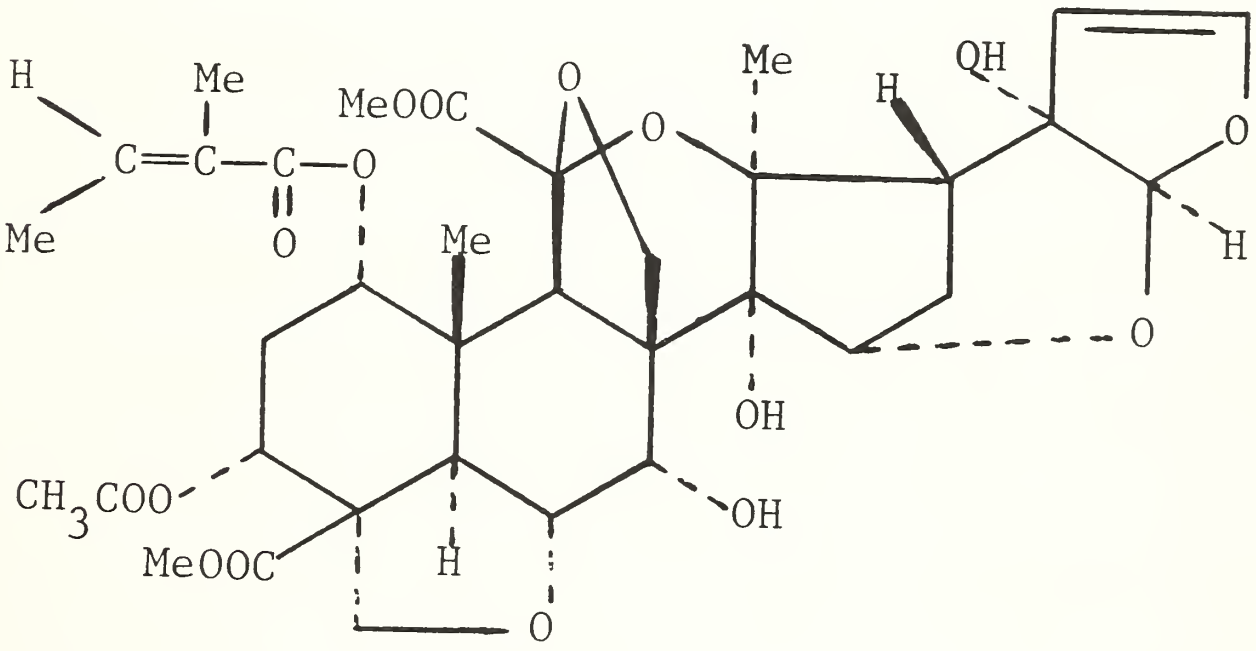

Figure 2.—Azadirachtin (14).

of A). Fraction 51-57 produced the same amount of antifeedant activity as an actual amount (0.35 p/m) of authentic azadirachtin; thus, the actual p/m = 0.35 for 51-57 rather than 5. This also was proven via 217-nm absorption and comparison of peak heights on hplc. Replication of the results in table 3 was also achieved through another hplc run and bioassay.

Thus, azadirachtin in neem seeds produces strong antifeedant activity in fall armyworm first-instar larvae. It deters feeding at 0.35 p/m in an artificial diet and causes an 88 percent reduction in weight of the first-instar larvae without mortality.

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